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Purification and characterization of human caseinomacropeptide produced by a recombinant *Saccharomyces cerevisiae*

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Abstract

Caseinomacropeptide (CMP) is a biologically active polypeptide derived from the C-terminal of milk κ-casein. CMP is heterogeneous since it is modified differently by glycosylation and phosphorylation after translation. Recently, recombinant human CMP (hCMP) has been produced as a secretory product in yeast. The present study aimed at the purification and characterization of recombinant hCMP. By sequential molecular cut-off ultrafiltration and anion-exchange chromatography, the recombinant hCMP in the culture broth could be purified to an HPLC purity over 94%. The authenticity of the purified hCMP was confirmed by sequence analysis of N-terminal amino acids. The recombinant hCMP was estimated to be 7.0 kDa by SDS–PAGE, and showed a lower glycosylation than the natural bovine CMP.

Keywords: Caseinomacropeptide; *Saccharomyces cerevisiae*; Ultrafiltration; Anion-exchange chromatography; O-glycosylation

Caseinomacropeptide (CMP)[1] is a glycopeptide of 64 amino acid residues (106–169) derived from the C-terminal of mammalian κ-casein [1]. It is released by the action of chymosin during the primary phase of milk clotting. CMP is known to have various biological functions such as the growth promotion of beneficial bacteria including *bifidobacteria* [2,3], the regulation of food intake [4,5], the depression of platelet aggregation, and the inhibition of the adhesion of oral *actinomyces* to cell membranes or of the binding of *cholera* toxin to its receptors [6]. CMP is heterogeneous due to different glycosylation and/or phosphorylation. Glycosidic residues attach to *Thr* and *Ser* by an O-glycosidic linkage during the post-translational modification in the Golgi apparatus of mammary gland cells [7]. The carbohydrate portion is made up of fucose, galactose, galactosamine, sialic acid, and other sugars.

The amino acid sequences of the CMP from seven species (bovine, zebu, water buffalo, sheep, pig, and human) have been reported [8]. Compared to other CMPs, human CMP (hCMP) contains more *Pro* residues and less carboxylic amino acids, and has a much higher carbohydrate content. In addition, hCMP is biologically more active than other CMPs. Azuma et al. [2] have reported that hCMP has a higher growth-promoting activity than bovine CMP (bCMP) by four times, and enhances the growth of *bifidobacteria* at a low

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[1] *Abbreviations used:* CMP, caseinomacropeptide; hCMP, human CMP; bCMP, bovine CMP.

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concentration of 50 ppm. Moreover, hCMP is more potent in the antithrombosis effect than bCMP [9]. Due to the several advantages above-listed as well as its human origin, hCMP has received more attention than other CMPs.

However, hCMP is still not commercially available. Recently, we constructed various recombinant yeast strains containing a chemically synthesized hCMP gene. Among them, *Saccharomyces cerevisiae*, with the hCMP gene under a galactose-inducible promoter, showed a high hCMP production of 2.5 g/L in a fed-batch fermentation (unpublished data). In this paper, we report the purification and characterization of the recombinant hCMP that was produced from a 500 L bioreactor culture of the recombinant *S. cerevisiae*. The glycosylation pattern of the recombinant hCMP is compared with that of natural bCMP and the differences are discussed.

Materials and methods

Strain and culture conditions

Saccharomyces cerevisiae 2805 (*MATα pep4::HIS3 prol-δ can1 GAL2 his3 δ ura3-52*) containing the recombinant plasmid pYEGα-hCMP was used for producing hCMP. The host *S. cerevisiae* 2805 is a uracil-auxotroph and has a mutation in a protease gene (*pep4*) to reduce the degradation of expressed foreign proteins [10]. The hCMP gene was chemically synthesized based on the amino acid sequence [6] and GenBank database (Accession No. M73628). For the construction of cloning vector, the hCMP gene was inserted into an episomal-type plasmid pYEGα (*GAL* promoter, *URA3* marker) [10] under the control of *GAL* promoter. The gene for α-factor secretion signal sequence was placed before the hCMP gene so that the recombinant hCMP was secreted in the culture medium. The expression vector, designated as pYEGα-hCMP, was transformed into *S. cerevisiae* 2805 by electroporation and the resulting recombinant strain was selected by its ability to grow on the uracil-deficient minimal medium.

The pre-inoculum culture was conducted in a 3 L baffled flask (working volume, 1 L) with a minimal selective medium containing 0.67% yeast nitrogen base (without amino acid), 0.02% histidine, and 2% glucose. The inoculum culture was conducted in a 50 L bioreactor (working volume, 25 L) with YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose), and transferred for main cultivation to be 7%(v/v) at the late exponential phase. The main culture was conducted in a 500 L bioreactor (working volume, 300 L) with YPDG medium (2% yeast extract, 2% glucose, and 3% galactose). The temperature and pH for the inoculum and main cultures were maintained at 30 °C and 5.5–5.7, respectively. The aeration rate in the main cultivation was 0.3 vvm and the agitation speed was 200 rpm.

Purification of recombinant hCMP

A culture broth of 1 L from the main cultivation was centrifuged at 5000*g* at 4 °C for 10 min to remove cells, and the supernatant was passed through a membrane filter (0.22 μm × 47 mm, Millpore, USA). The filtrate was applied sequentially to two molecular cut-off membranes (Pall Filtron, USA) of 100 and 30 kDa to remove large proteins and small proteins, respectively. The concentrated solution (40 mL) from the 30 kDa molecular cut-off was loaded onto an anion-exchange column chromatograph containing 300 mL of diethylaminoethyl cellulose resin (DE52, Whatman, USA). The column was washed with 20 mM Tris–HCl buffer at pH 7.5, and the bound proteins were eluted with a linear gradient of 0–1 mM NaCl in the same buffer at 1 mL/min. The separated compounds were monitored at 214 and 280 nm.

Analyses

The cell concentration was measured spectrophotometrically at 660 nm (Lambda 20, Perkin–Elmer, USA). An optical density of 1.0 corresponds to the dry cell mass of 0.24 g/L. Glucose, galactose, and ethanol in the culture supernatant were analyzed using a high-performance liquid chromatograph (HPLC) (1100 series, Agilent Technologies, USA) equipped with a Shodex-SH1011 packed column (Ø 8 mm × 300 mm, Showa Denko K.K., Japan) and a refractive index detector. The column temperature was 50 °C and an aqueous solution of 0.01 N H_2SO_4 was used as an eluent at 0.6 mL/min.

The amount of recombinant proteins secreted to the culture broth was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 15%). Protein bands in the gel were quantified by image analysis after silver nitrate staining. The hCMP was also analyzed by a reverse-phase HPLC (1100 series, Agilent Technologies, USA) on a C18 column (5 μm, Ø 4.6 mm × 250 mm, Hi-pore, BioRad, USA) at 30 °C with a UV detector at 214 nm. Eluting solutions were composed of acetonitrile, double distilled water, and trifluoroacetic acid; mobile phase A was in the ratio of 100:900:1(v/v/v) and mobile phase B, 900:100:0.8(v/v/v). The elution was performed by increasing mobile phase B from 0 to 50% while maintaining a constant flow rate of 1 mL/min. The purity and amount of hCMP during the purification step were estimated based on the relative peak area of the HPLC chromatograms. The total protein was determined by the Bradford method [11].

To analyze the N-terminal amino acid sequences, the purified hCMP was run on a 15% SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The band corresponding to hCMP was excised and sub-

jected to a protein sequencer (Procise 491, Applied Biosystems, USA).

The glycosylation of the recombinant hCMP was analyzed by glycostaining and HPLC analyses. Commercially available bCMP (C7278, Sigma, USA) was used as a control since the authentic hCMP was not available. Glycostaining was conducted by periodic acid–Schiff (PAS) staining in an SDS–PAGE [12]. The deglycosylation of the recombinant hCMP was conducted by treatment with *O*-glycosidase (G1163, Sigma, USA), whereas that of bCMP was by treatment with both *O*-glycosidase and sialidase (N8271, Sigma, USA). According to the manufacturer's certificate (Sigma, USA), the commercial bCMP contained about 13% sialic acid in its sugar moiety and this inhibits the action of *O*-glycosidase [13]. The conditions for HPLC analyses for the CMPs after deglycosylation were the same as those described before.

Results and discussion

Production of recombinant hCMP

Fig. 1 shows the result of the large-scale fermentation in the 500 L bioreactor (working volume, 300 L) for the production of hCMP by recombinant *S. cerevisiae*. The fermentation was conducted in a batch mode at 30 °C and pH 5.5–5.7. Glucose was depleted at around 18 h, and galactose consumption followed. The final cell concentration was 28.2 g/L at 70 h. Since the *GAL* promoter is repressed in the presence of glucose, the production of hCMP started when the glucose was depleted (Fig. 1B). The production of the recombinant hCMP in the culture broth was approximately 110 mg/L (refer to Table 1), which was much lower than that of a fed-batch 5 L bioreactor fermentation (2.5 g/L). This is attributed mainly to the mode of reactor operation: a batch mode was employed in the present study, whereas a fed-batch mode was used in the 5 L bioreactor with an optimal feeding strategy for glucose and galactose. It should be noted that process optimization is very important in recombinant fermentation scale-up.

Table 1
Purification summary of recombinant hCMP[a]

Purification step	Total protein (mg)	Purity[b] (%)	hCMP[c] (mg)	Recovery yield (%)
Culture supernatant	500	22	110	100
Ultrafiltration	226	44	100	91
Anion exchange	50	94	47	43

[a] Purification was started with 1 L of culture broth.
[b] Purity was estimated based on percentage peak areas of HPLC chromatogram.
[c] hCMP amount was calculated based on purity of hCMP to total protein.

Purification of recombinant hCMP

Fig. 2 shows the results of the sequential ultrafiltration of the culture broth. When filtered through a 100 kDa molecular cut-off membrane, most hCMP and some large proteins appeared in the filtrate (Fig. 2, lane 4). When this filtrate was passed through a 30 kDa cut-off membrane, most hCMP were found in the retentate (Fig. 2, lane 5). The total protein in the initial culture supernatant of 1 L was about 0.5 g, whereas the protein in the 30 kDa retentate (40 mL) was about 0.23 g.

Considering that the molecular weight of hCMP is 7.0 kDa, the result of ultrafiltration, that most recombinant hCMP were obtained between 30 and 100 kDa, is extraordinary. Kawasaki et al. [14] have reported that, at pH 6.5, bCMP is rejected by a 50 or 20 kDa cut-off mem-

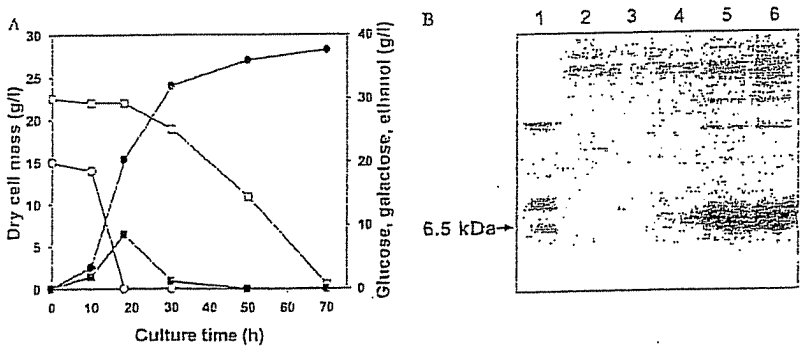

Fig. 1. Cell growth and hCMP production in large-scale culture (500 L) of recombinant *S. cerevisiae* containing pYEGα-hCMP. (A) Dry cell mass (●), glucose (○), galactose (□), and ethanol (■). (B) SDS–PAGE analysis: lane 1, molecular weight standard; lane 2, host (without recombinant plasmid); lane 3, before induction; lanes 4–6, culture broth supernatant at 30, 50, and 70 h.

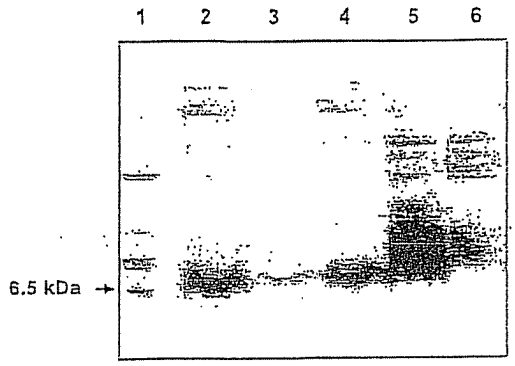

Fig. 2. SDS–PAGE during sequential ultrafiltrations. Lane 1, molecular weight standard; lane 2, culture supernatant; lanes 3 and 4, filtrate of 30 and 100 kDa; lanes 5 and 6, retentate of 30 and 100 kDa.

brane, whereas it is mostly permeated through the same membranes at pH 3.5. They suggested that the bCMP associates at a neutral pH through non-covalent interaction between bCMP molecules. An alternative explanation has been proposed by Minkiewicz [15], who has reported that the high negative charge of bCMP causes internal electrostatic repulsion, resulting in a larger volume than other protein molecules of a similar size. In this study, the culture pH at harvest was about 5.5 and was not adjusted before ultrafiltration. Therefore, the apparently high molecular weight of the recombinant hCMP during the ultrafiltration seemed to be related to the protein association and/or large volume.

The retentate from the 30 kDa cut-off membrane was loaded onto an anion-exchange chromatography column packed with diethylaminoethyl cellulose for further purification. Fig. 3A shows the elution profile with a linear gradient of NaCl at two wavelengths, 214 nm for all amino acids and 280 nm for aromatic amino acids. Because of the absence of aromatic amino acids, CMPs can be identified by the lack of absorbance at 280 nm [1]. As shown in Fig. 3, one major and two minor peaks were obtained between 0.3 and 0.5 mM of NaCl at 214 nm. The first eluting peak showed a strong absorbance at 214 nm but a weak absorbance at 280 nm. Two minor peaks showed a strong absorbance at both 214 and 280 nm. Therefore, the fractions corresponding to the first peak were selectively collected and analyzed by SDS–PAGE. As shown in Fig. 3B, a single and homogeneous band was obtained at around 7.0 kDa. The N-terminus amino acid sequences of the purified bCMP were found to be Ile-Ala-Ile-Pro-Pro, which are identical to those deduced from DNA sequences of hCMP. In Fig. 3B (lane 3), the commercial bCMP was also run for comparison. Although the molecular weights of both CMPs are about the same at 7.0 kDa, bCMP migrated much less and its molecular weight was estimated to be 20 kDa.

Table 1 summarizes the purification procedures. The recovery yield and purity were about 43 and 94%, respectively. From the relatively simple processes of ultrafiltration and anion-exchange column chromatography, an electrophoretically pure CMP was obtained.

Glycosylation analysis

Fig. 4 shows the PAS staining and mobility of the recombinant hCMP before and after deglycosylation. The results for the natural bCMP are also given in comparison. The recombinant hCMP, both untreated and treated by *O*-glycosidase, showed a weak staining (Fig. 4A, lanes 1 and 2). Mobility was also about the same regardless of enzymatic treatment (Fig. 4B, lanes 2 and 3). In contrast, in case of the natural bCMP, significant differences were observed before and after deglycosylation; the untreated bCMP exhibited a stronger staining (Fig. 4A, lanes 3 and 4) and a reduced mobility (Fig. 4B, lanes 4 and 5) than that of the treated bCMP. This indi-

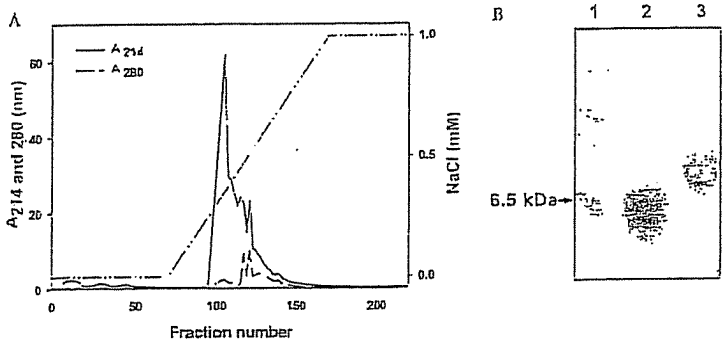

Fig. 3. Purification of recombinant hCMP. (A) Elution profiles at 214 (—) and 280 nm (- - -) from anion-exchange chromatograph with NaCl gradient (----). (B) SDS–PAGE analysis: lane 1, molecular weight standard; lane 2, purified recombinant hCMP; lane 3, natural bCMP.

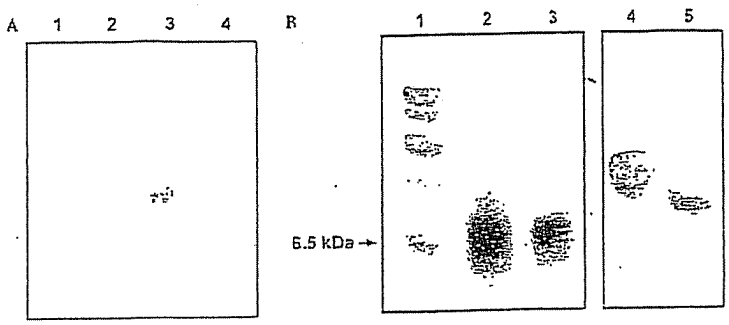

Fig. 4. Glycosylation analysis of the purified recombinant hCMP and natural bCMP. (A) Total carbohydrate was analyzed by 15% SDS–PAGE with PAS staining: lane 1, hCMP; lane 2, deglycosylated hCMP; lane 3, bCMP; lane 4, deglycosylated bCMP. (B) Mobility shift analysis on 15% SDS–PAGE with silver nitrate staining: lane 1, molecular weight standard; lane 2, hCMP; lane 3, deglycosylated hCMP; lane 4, bCMP; lane 5, deglycosylated bCMP.

cates that the level of glycosylation in the recombinant hCMP produced by *S. cerevisiae* is much lower than that of the natural bCMP.

The less glycosylation of the recombinant hCMP is attributed to the difference in the sequence specificity of O-glycosylation between yeast and mammalian cells during its production [16–18]. In mammalian cells, glycosylation at *Thr* or *Ser* residue is promoted when *Pro* is present nearby; whereas, in yeast, glycosylation is strongly inhibited when *Pro* is located immediately after the glycosylation site or an acidic amino acid is present in a close vicinity. hCMP has many acidic residues (11%) as well as *Pro* (18%) residues, some of which are located immediately after *Thr* or *Ser*, and this seemed to greatly reduce the glycosylation level in the recombinant yeast (GenBank Accession No. M73628).

HPLC analyses of hCMP and bCMP

Fig. 5 shows HPLC chromatograms of the recombinant hCMP and natural bCMP before and after deglycosylation. The purified hCMP exhibited a simple chromatographic profile with a single major peak at 18.9 min (Fig. 5A). This chromatographic profile did not change after the enzymatic deglycosylation. In comparison, the natural bCMP showed many peaks between 20 and 30 min, indicating that it might be heterogeneous in glycosylation pattern (Fig. 5B). Especially, the early peaks eluted before 23 min could be assigned to glycosylated forms, since they were shifted into 24.5 min after the enzymatic treatment. These results, along with the previous ones shown in Fig. 4, confirm that the extent of glycosylation in the natural bCMP is significantly higher than that of recombinant hCMP. Fig. 5 also shows that recombinant hCMP has a shorter retention time than natural bCMP. Amino acid sequences between hCMP and bCMP are reported to diverge by 45% [8], and this seems to affect the retention time.

In summary, purification and characterization of recombinant hCMP produced from *S. cerevisiae* was studied. Recombinant hCMP was purified about 94% by two sequential ultrafiltrations and an anion-exchange chromatograph. The recombinant hCMP appeared to be

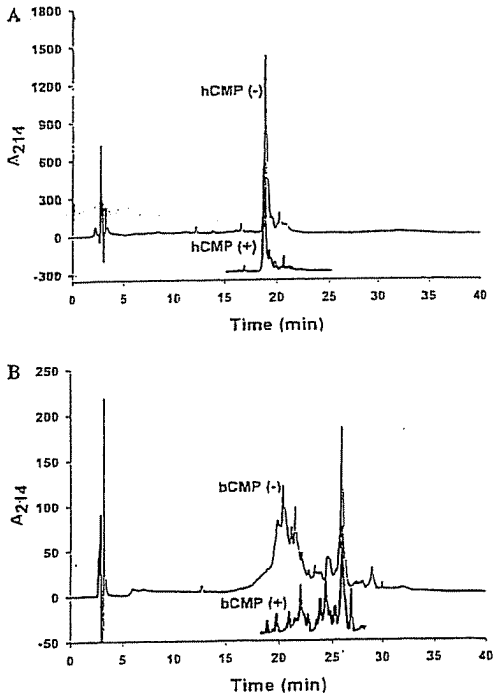

Fig. 5. Reverse-phase HPLC chromatograms. (A) Recombinant hCMP before (–) and after (+) deglycosylation. (B) Natural bCMP before (–) and after (+) deglycosylation.

significantly less glycosylated than the natural bCMP. Since the biological activity of many glycoproteins often depends on the content and structure of carbohydrate moieties attached to peptide, the antiobesity activity of recombinant hCMP is under investigation.

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